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# *In Vivo* Evaluation of a Tri-Phasic Composite Scaffold for Anterior Cruciate Ligament-to-Bone Integration

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*Abstract*—**The widespread clinical implementation of hamstring tendon (HT) autografts for anterior cruciate ligament (ACL) reconstruction is currently limited by the unpredictable integration of the graft with subchondral bone and a lack of devices that are capable of promoting biological fixation of HT grafts to bone. The site of HT graft fixation within the bone tunnel has been identified as the weak point in the reconstructed ACL, likely due to the failure of the graft to reestablish the physiological tendon-bone interface capable of transmitting load from the ligament to bone while minimizing stress concentration at the interface. Although a fibrovascular tissue has been shown to form at the graft-bone interface, this fibrovascular tissue is non-anatomically oriented compared to the native fibrocartilage found at direct ligament to bone insertions. Interface tissue engineering embodies a new approach for graft fixation, focusing on securing tendon grafts to bone via biological fixation wherein the complex functional interface found natively at tendon and ligament junctions with bone are regenerated at the graft insertion site into the bone tunnels. This study focuses on the *in vivo* evaluation of a novel biomimetic, triphasic scaffold system co-cultured with relevant cell types found at the graft-bone interface, specifically fibroblasts, chondrocytes, and osteoblasts. The scaffold is intended to promote biological fixation of HT grafts to bone by guiding the reestablishment of an anatomically-oriented and mechanically functional fibrocartilage interfacial region. It was found that the cell-seeded triphasic scaffolds supported cellular interactions as well as tissue infiltration and abundant matrix production *in vivo.* In addition, controlled phase-specific matrix heterogeneity was induced on the scaffold, with distinct mineral and interface-like tissue regions. The results of this study demonstrate the feasibility of multi-tissue regeneration on a single graft, as well as the potential of interface tissue engineering to enable the biological fixation of soft tissue grafts to bone.**

## I. Introduction

THE anterior cruciate ligament (ACL) is the most commonly injured ligament of the knee, with approximately 100,000 reconstruction procedures performed each year in the United States [1-3]. The long term performance of ACL reconstruction grafts is dependent in part on graft fixation [4,5]. Autologous hamstring tendon-based grafts are increasingly utilized for ACL reconstruction due to the high incidence of donor site morbidity associated with bone-patellar tendon-bone grafts [6,7]. Clinically, the soft tissue grafts are secured using mechanical fixation devices, which may restore the physiological range of motion and joint function; however, biological fixation is not achieved since the native fibrocartilage insertion site fails to be regenerated [8]. Without a functional biological interface, the graft-bone junction exhibits limited mechanical stability [4,5,9], and the lack of graft integration constitutes the primary cause of graft failure [4,5,10-12].

The ACL inserts into bone through a characteristic fibrocartilage interface, with controlled spatial variation in cell type and matrix composition. Three distinct tissue regions are observed: ligament, fibrocartilage (non-mineralized and mineralized) and bone [13-15]. It is believed that this controlled matrix heterogeneity permits a gradual transition of mechanical load between soft tissue and bone, and in turn minimizes the formation of stress concentrations [14,16]. Since the degree of graft integration is a critical factor governing the clinical success of ACL reconstructions, we believe interface regeneration will significantly improve the long term outcome through biological integration of soft tissue-based grafts with bone. However, the mechanisms governing interface regeneration are not well understood. While tendon-to-bone healing following ACL reconstruction with semitendinosus grafts does not lead to the re-establishment of the native insertion, a fibrovascular tissue is consistently formed within the bone tunnels [8,17]. These observations suggest that interaction between cells derived from tendon (i.e. fibroblasts) and bone tissue (i.e. osteoblasts) may play a role in interface regeneration. Previous work from our laboratory examining the interaction of osteoblasts and fibroblasts in monolayer co-culture revealed that co-culture modulated cellular phenotype, resulting in a decrease in osteoblast alkaline phosphatase activity and the expression of type II collagen, accompanied by an increase in the mineralization potential of fibroblasts [18]. In addition, Nawata *et al.* reported that post-natal reorganization of the ACL-to-bone insertion may involve transdifferentiation of fibroblasts into fibrochondrocytes [19].

Based on these previous findings, it is clear that interface regeneration will require controlled cell-to-cell interactions. Therefore, our approach is to regenerate the interface through biomimetic scaffold design and the co-culture of relevant cell types on the scaffolds. In a previous study

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[20], we designed a multi-phasic scaffold with three distinct phases mimicking the tissue organization found at the native ACL-to-bone insertion, and performed a co-culture of fibroblasts and osteoblasts on this novel scaffold system. Distinct cellular and matrix regions were maintained on the three phases of the scaffold, and both cell types migrated into direct contact in the middle phase.

The current study describes the *in vivo* evaluation of this triphasic scaffold tri-cultured or co-cultured with ACL fibroblasts, chondrocytes, and osteoblasts. Specifically, we evaluated phase-specific maintenance of distinct tissue regions, scaffold integrity, and the effects of the *in vivo* environment on scaffold mechanical properties over a two-month period. It was anticipated that the *in vivo* co-culture would result in distinct matrix regions and improved mechanical properties of the scaffold.

## II. Materials and Methods

### A. Scaffold Fabrication

The continuous tri-phasic scaffold (Figure 1) consists of three distinct phases: Phase A for soft tissue, Phase C for bone, and Phase B for interface development. Each phase was designed with optimal composition and geometry suitable for the tissue type to be regenerated. Phases A, B, and C consist of poly(lactide-*co*-glycolide) (PLGA,10:90) knitted mesh, PLGA (85:15) microspheres, and PLGA(85:15)/Bioactive Glass (45S5,BG) composite microspheres, respectively. The microspheres were formed via a double emulsion method [21], and continuous triphasic scaffolds were formed by sintering above the polymer $T_g$.

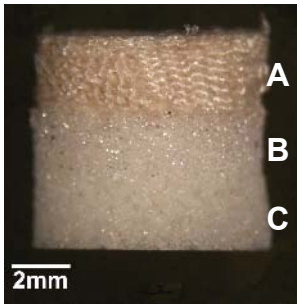


*Figure 1. Tri-phasic scaffold with Phase A for soft tissue, Phase B for interface development, and Phase C for mineralized tissue.*

### B. Tri-Culture and Co-Culture on the Scaffolds

Bovine osteoblasts (OB) and ACL fibroblasts (FB) were obtained by explant culture, and chondrocytes (CH) were isolated from articular cartilage by enzymatic digestion. FB ($5x10^5$ cells/scaffold) and OB ($2.5x10^5$ cells/scaffold) were seeded onto Phases A and C, while CH ($5x10^5$ cells/scaffold) were loaded into Phase B in 0.5% agarose (Sigma, MO). Three groups were investigated: FB+CH+OB (tri-culture), FB+OB (co-culture), and acellular.

### C. In Vivo Implantation

All animal procedures were performed in accordance with a protocol approved by the institutional animal care and use committee. Following 3 days of *in vitro* culture, scaffolds were implanted into the dorsa of male athymic rats (NIH-rnu, Charles River Laboratories, MA). Three scaffolds were inserted into separate subcutaneous pouches in each rat, and incisions were closed with sutures. Rats were sacrificed at 2, 4, and 8 weeks. At each time point, the mechanical properties, cell proliferation and infiltration, tissue production, and mineralization were evaluated.

### D. Cell Proliferation and Tissue Production

Cell proliferation and infiltration were evaluated by measuring total DNA per scaffold using the PicoGreen dsDNA fluorometric assay (Molecular Probes, OR). The cells in each scaffold were lysed with 0.1% Triton-X solution (Sigma, MO) and the scaffolds homogenized before following the manufacturer's recommended protocol. Tissue production and infiltration were evaluated by histological and immunohistochemical analyses. Scaffolds were fixed in 10% neutral buffered formalin for 48 hours immediately following explantation. Half of the scaffolds were embedded in paraffin and sectioned for histological staining. Collagenous tissue formation was evaluated with a modified Goldner Masson trichrome stain, and glycosaminoglycans were imaged using alcian blue stain with a pH of 1.0 [22]. Type I and type II collagen content was evaluated by immunohistochemistry. The remaining half of the scaffolds were analyzed with μCT, then plastic embedded in poly(methyl methacrylate). Plastic sections were stained with von Kossa silver nitrate solution in order to image scaffold mineral content.

### E. Scaffold Mechanical Properties

The mechanical properties of the scaffolds were determined by uniaxial compression testing (n=5, MTS 810, MN). Scaffolds were preloaded up to 10 N, and compressed at a rate of 1.3 mm/min. Compressive modulus was calculated by finding the slope of the initial linear regions of the stress-strain curve, and yield strength was calculated by finding the stress at a 0.1% offset from the linear region. Results are presented in the form of mean ± standard deviation. A two-way analysis of variance (ANOVA) was performed to determine seeding and temporal effects on scaffold mechanical properties. Fisher's LSD post-hoc test was performed for all pair-wise comparisons, and statistical significance was attained at $p<0.01$ (Statistica, release 5.5, StatSoft, Inc., OK).

## III. Results

### A. Tissue Ingrowth and Matrix Distribution

Total cell number increased in all groups tested after initial seeding. A drop in cell number was observed from weeks 4 to 8 most likely due to tissue remodeling and rapid hydrolytic degradation and resorption of Phase A, although a higher cell number was found in the tri-culture and co-culture groups compared to the acellular group at week 8 (data not shown). An extensive collagen-rich matrix was prevalent in the three phases of the seeded scaffolds, and was considerably more abundant than in the acellular group at weeks 4 and 8 (Figure 2). A type II collagen and glycosaminoglycan-rich region was found only in the tri-

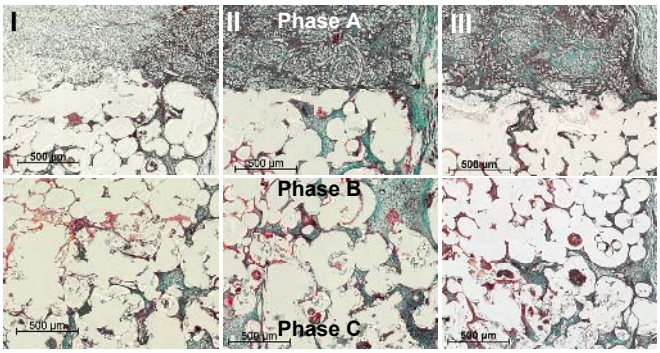


*Figure 2. Collagenous tissue formation (green) in the triphasic scaffolds was significantly greater in the co-culture and tri-culture groups compared to the acellular scaffolds. Modified Goldner Masson Trichrome stain of I) Acellular, II) Co-culture, and III) Tri-culture scaffolds after 4 weeks in vivo (5x, bar = 500 µm).*

culture group, and this cartilaginous region, found between Phase A and Phase B, had a cell distribution resembling that of neonatal interfacial fibrocartilage tissue (Figure 3). A zonal distribution of mineral was found, with mineralized matrix shown to be confined to Phase C for all groups and time points by µCT and von Kossa histological analyses (Figure 4).

### *B. Scaffold Mechanical Properties*

Compression testing of scaffolds indicated an initial decrease in compressive modulus due to hydrolytic polymer degradation. This initial drop was followed by a significant increase in modulus in the co-culture group between weeks 4 and 8 (Figure 5). A corresponding increase was also observed in the tri-culture group, although this difference was not significant. A lower modulus was found in the acellular group at week 8, most likely due to less extensive tissue formation and mineralization compared to the seeded groups. There was a generally decreasing trend in compressive yield strength over the eight-week period; however, yield strengths for the tri-culture and co-culture groups were higher than for the acellular group at week 8, although these differences were not significant (data not shown).

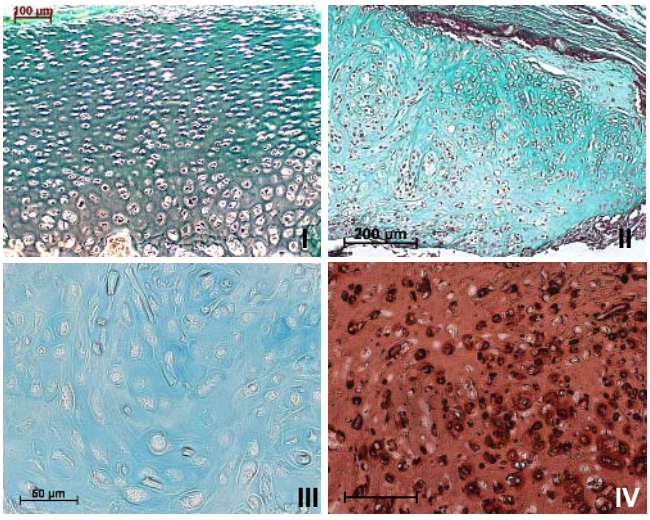


*Figure 3. Fibrocartilage-like chondrocyte tissue region found in Phase A of the tri-culture scaffolds at week 8 resembles neonatal ACL insertion fibrocartilage tissue. Modified Goldner Masson Trichrome stain of I) neonatal insertion fibrocartilage (10x, bar = 100 µm), and II) chondrocyte tissue region in the tri-culture group (10x, bar = 200 µm) showing cell morphology and distribution as well as high collagen content (green). III) Alcian blue stain demonstrating GAG content (blue, 32x, bar = 50 µm). IV) Immunohistochemical stain indicating the abundance of type II collagen in this tissue (red, 20x, bar = 100 µm)*

## IV. Discussion

In this study, we evaluated the effect of the *in vivo* environment on the tri-culture of ACL fibroblasts, chondrocytes, and osteoblasts on a novel tri-phasic scaffold. We found that the biomimetic, tri-phasic scaffold supported cell growth and cell-specific matrix production *in vivo*, with abundant collagenous tissue found in the co-culture and tri-culture groups. The results demonstrate that *in vitro* cell seeding enhanced *in vivo* matrix production. This finding suggests that matrix elaboration is a combination of tissue ingrowth and cell-mediated matrix production. In addition, an increase in compressive modulus was found in the seeded groups, suggesting that cell interactions and matrix elaboration are important for maintaining structural integrity and phase continuity.

A fibrocartilage-like matrix was found in the tri-culture group. This matrix contained type I collagen and was rich in type II collagen and proteoglycans. In order to localize this region in Phase B where intended instead of Phase A as observed in this study, the scaffold will be optimized, particularly scaffold porosity, for future studies. In addition, a phase-specific distribution of mineralized matrix was found to be confined to Phase C for all scaffold groups and time points, demonstrating the osteointegrative and osteoconductive potential of this phase of the scaffold, which is intended to support the growth of mineralized tissue within the bone tunnel. The findings of this study collectively demonstrate the potential for multi-tissue regeneration on a single continuous scaffold mimicking the native interface matrix distribution and organization for promoting biological fixation of soft tissue grafts to bone.

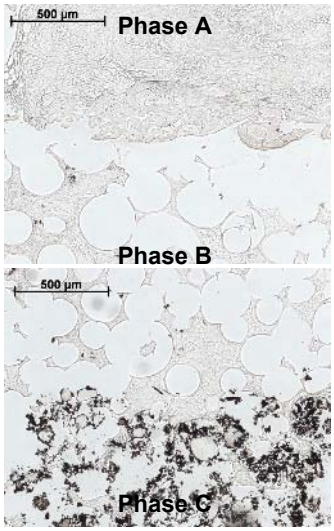


*Figure 4. Zonal distribution of mineral in tri-culture scaffold at week 4 as shown by von Kossa histological stain. Mineral (black) was confined to Phase C of all scaffold groups at all time points.*

It is likely that in this study the full *in vivo* response may not have been observed in the subcutaneous, athymic rat model, especially when compared to an intra-articular, immunocompetent

animal model. In particular, scaffolds were not loaded in the subcutaneous environment, and implantation in a weight bearing site may influence cell response. Studies are underway to evaluate the scaffold *in vivo* in a loaded intra-articular model.

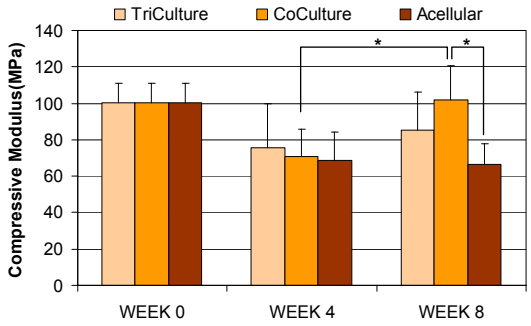


*Figure 5. Compressive modulus of scaffolds over time in vivo. Tissue formation and mineralization compensated for the initial decrease in compressive modulus in the co-culture and tri-culture group. An increase in modulus was not seen in the acellular group (* $p<0.01$).*

## V. Conclusion

We have reported here the *in vivo* evaluation of a novel triphasic scaffold for soft tissue graft-to-bone integration. The biomimetic, triphasic scaffold supported heterotypic interaction of interface-relevant cells, as well as tissue infiltration and abundant matrix production. It was found that *in vivo* tissue ingrowth, matrix production, and mineralization compensated for scaffold degradation. In addition, controlled phase-specific matrix heterogeneity was induced on the scaffold, with a distinct mineral region crucial for osteointegration, and an interface-like tissue in the tri-culture group. Future studies will focus on scaffold optimization and *in vivo* scaffold evaluation in a tendon-to-bone healing model.

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